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ANTIOXIDANT ENZYMES OF DIOSPYROS FERREA (WILLD), BAKH LEAF

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ABSTRACT

Objective: To evaluate the antioxidant activity of Diospyros ferrea, Ebenaceae family methanolic leaf extract.

Methodology: Huge spectrum of antioxidant molecules were identified in the leaf extracts of Diospyros ferrea worked through GC-MS studies. β-Sitosterol, Betulinic acid, Betulin, Lupeol, Ursane, Oleanane derivatives are triterpenoids whereas flavonoids are naphathaquinones which are phenolic compounds beneficial for the inhibition of reactive oxygen species. Total phenolic content (mg GAE/g extract) and enzymes SOD (superoxide dismutase), Catalase (CAT), Glutathion (GTH) properties were investigated by the DPPH (diphenyl-1-picrylhydrazyl) using UV spectrophotometry at 517nm. Lipid peroxidation -LPO inhibition activity (free radical effect) was also by studying the liver antioxidant enzymes in diabetic rats.

Results: Total phenolic content in Hexane 37.54 ± 2.30, Ethyl acetate 42.86 ±1.75 and Methanol 55.03±0.42mgGAE/g extract. DPPH-1,1-diphenyl-1-picrylhydrazyl at 50µg/ml 100 & 150µg/ml were 60±0.17, 64±0.14, 68±0.33 µg/ml and SOD 27±0.11, CAT 36±0.19, GSH 49±0.63 µg/ml compared with standard (70±0.1 & 58) respectively. Calculation of DPPH% of antioxidant activity 40.1220, absorbance at 517nm, animals treated with 200 mg/kg and 400 mg/kg doses of D.ferrea leaf extract enzymes manifested in diabetic rats 89.83±01.302 and 94.5±0.670 IU/L levels were significant ($p < 0.001$) in LPO.

Conclusion: Results indicate that leaf extract has an efficient phenol activity with significant increase in the antioxidant levels to nullify the oxidative stress in the tissues. Further studies are necessary to explore the drug property of leaf material of *D.ferrea*.

KEYWORDS: Antioxidants, Phenols, DPPH, Enzymes SOD, CAT & GSH.

INTRODUCTION

Accumulation of free radicals in excess quantity causes imbalance during metabolic activities that can be prevented by the defense molecules called antioxidants abundant in plant materials. They are enzymatic antioxidant weapons -Superoxide dismutase (SOD) Catalase (CAT), GPx-Glutathion peroxidase and non-enzymatic antioxidants like flavonoids, glutathione, carotenoids, ascorbic acid and tocopherol. More reactive oxygen species like superoxide anion radicals, hydroxyl and hydrogen peroxides released under stress conditions than enzymatic antioxidants.

Principle ROS radicals are always ready to detoxify the molecules by releasing the certain enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in order to maintain homeostasis. Superoxide anion radical is metabolized by the metalloenzymes superoxide dismutase to form hydrogen peroxide and molecular oxygen. The copper zinc containing SOD are highly stable enzymes and can easily isolated in all eukaryotic cells and iron and manganese containing SOD in prokaryotic cells which is cytotoxic. Electrical neutrality in the tissues were maintained by the superoxide dismutase (SOD) which catalyzes the superoxide radical by dismutation reaction, whereas catalase(CAT), glutathione reductase(GR), glutathione peroxidase(GPx) are responsible.

Main bioactive compounds are phenols, as they have an aromatic ring that allows the stabilization and relocation of the unpaired electrons in their structure, facilitating the donation of hydrogen atoms and electrons naturally from their hydroxyl groups (Rice Evans *et al.,* 1997). Antioxidant activities were assayed by 2 different methods, ABTs (quantification of free radical scavenging capacity) and FRAP (quantification of compounds capable of reducing the complex ferric ions to ferrous complex (Biskup et.al.,2013). Phenolic compounds act as chelating metals of iron and copper, breaking free radicals chain reactions and also accelerating the enzyme activity.

To prevent oxidant damage, cells have involved to secrete antioxidant enzymes such as SOD, GPx-Glutathion peroxidase and CAT and Potential antioxidants are Phenolics, flavonoids, anthocyanins, tocopherols, α-tocopherol and Phytol isoprenoid alcohol usually join with Superoxide dismutase-SOD (Kakkar *et al* 1983) and Glutathione enzymes. Phenols and Flavonoids have been frequently correlated with the antioxidant potential

showing strong capacity to eliminate free radicals in the blood serum and promotes phagocytosis through enzymes SOD, GSH, and CAT.

Phenols & Phenolic acids are hydroxylated derivatives of benzoic acids and esters of glucose whereas flavonoids are Amyrins, Tannins & Coumarins, a class of polyphenolic compounds exert protective effects against oxidative stress. The phenol and flavonoid play an important role in preventing diseases as they adsorb and neutralizes free radicals (Pandey & Rizvi 2009). According to Nabavi et.al 2009, the free radicals are reactive oxygen species generated in our body which are harmful to create inflammation and cancer aging. The isolated di-naphtho-diospyrols show promising antioxidants in a dose dependent manner which exhibits excellent antioxidant and free radical scavenging activity (Zhang *et.al* 2012).

Most biologically active class of phenolic antioxidants could inhibit lipid self-oxidation by scavenging free radicals and are reacting with singlet oxygen molecules screened by DPPH method 1,1- diphenyl-1-picrylhydrazyl. As SOD was a unique and valuable enzyme to inhibit radical reactions by reducing iron ions to superoxidases. Due to oxidative stress the enzyme Superoxide dismutase-SOD and branched amino acids when not released in sufficient quantity, naturally cells cannot catabolize carbon sources leads to mutagenesis. The DPPH radical scavenging assay proves that methanol extract had potential effect on inhibiting the free radicals in dose dependent manner. C & E vitamins are essential enzyme co-factors which nourishes cells and cellular components (Mallavadhani 1998, Maridass 2008).

Catalase, a heme-iron containing protein located on peroxisomes of tissues which helps to remove hydrogen peroxide. It consists of four catalase subunits as alpha -Cytosol and Xanthane in liver and in RBC- Glutathione peroxidase and Glutathione-S-transferase. Glutathione is the most effective tripeptide antioxidant, which is a mixture of glycine, cysteine, and glutamic acid amino acids. Potential source of Glutathion helps to detoxify the alkylated agents with –SH group and neutralize the detoxification of xenobiotics and free radicals.

Lipid Peroxidase is the best free radical mediated enzyme for biological membranes in association with several pathological, physiological and toxicological reactions leads to DNA damage (Zhang, C Li 2013), Ferritins and transferrin forms a complex with iron ions enable to reduce LPO. The scavenging DPPH radical is a common method to evaluate the antioxidant activity. The DPPH radical is a relatively stable radical that does not exist in nature but the deep purple colour of the radical monitored with a Spectrophotometer (Ranjan K et.al., 2023).

Maridass 2008, have shown enormous medicinal values with antioxidant capacity in *D. peregrine* Gurk and *D. malabarica* Desr species with whole plant. The total phenolic content (TPC) in persimmon (*D.melanoxylon*) was significantly high i.e., 112±2.89 mg of GAEs/g of extract and high phenol content (223.5±0.26 mg GAE/g) was reported from Sirisha et.al 2018. Reports on low phenolic value ranged from 0.434 mg GAE/g to 12.87 mg GAE/g (Moniruzzaman et al. 2019). The total flavonoid content (TFC)of persimmon fruit was 6.97±0.07 mg QAEs/g of extract and according to Rehman et al 2020 lower value to flavonoid (64.7±7 mg of GAEs/100g).

Diospyros ferrea

D.ferrea leaves are simple, alternate and distichos, about 1,5-7 cm in length. Shape is oblong or elliptic or obovate. Apex is acute, base is attenuate or slightly rounded. Margin is entire, blade is coriaceous, glabrous on both sides. Midrib of the leaf is flat above. The leaf shown in moisture form and was removed by shade dried, after that the fragments were pulverized into a coarse powder.

MATERIAL & METHODS

Collection of plant material:

The leaf material of *Diospyros ferrea* plant was collected from the hill slopes of Seshachala forest, Tirupathi, Andhra Pradesh, South India. Taxonomic authentication was given by Mr. A. Ravi Kiran, BSI, Coimbatore, India and a voucher specimen is deposited in Department of Botany, Acharya Nagarjuna University, Guntur and the specimen number is ANU Y9B0R025. The collected leaf was shade dried till the moisture content was evaporated and finally pulverized into small pieces.

Preparation of Soxhlet Extraction:

Soxhlet extraction required where the desired compound has a limited solubility in a solvent and impurities in that solvent. Shade dried and healthy fresh leaves of *D.ferrea* were subjected to selective extraction 100 g of leaf material was uniformly packed in the thimble and extracted with 300 ml of methanol. The process continued till the solvent in siphon tube has become colorless. The obtained filtrate was condensed through rotary evaporator at 45°C and preserved at 4°C in airtight bottles for further use.

The leaf shown in moisture form and was removed by shade dried, after the fragments and pulverized into a coarse powder. Fifty grams of the powdered material was soaked in 300 mL of polar solvent methanol. The plant leaf was extracted with Hexane, ethyl acetate, methanol and stored in refrigerator at 4°C until use extract.

Fig 2. Shade dried leaf material of *D. ferrea*

Experimental Animals:
Healthy male Wistar rats of weight 200–250 g /bw, were used for the study and were housed in polypropylene (55x32.7x19 cm) cages and acclimatized before the experiment for 14 days under standard laboratory conditions (12/12 h light and dark cycles) with temperature of 23°C ± 2°C and humidity (60 ± 10%) with food and water ad *libitum*.

Experiment at Albino Research labs, Hyderabad

1.Estimation of total phenolic content:
The total phenolic contents in medicinal plants were determined spectrophotometrically by Folin-Ciocalteu method (Singleton et al.,1965,1999). Gallic acid was used to set up the standard curve. The content of phenolic compounds of the samples was expressed as gallic acid equivalents (GAE) in mg per gram dry weight. All the samples were analyzed in triplicates.

Principle: Phenols react with phosphomolybdic acid in Folin's reagent to produce a blue colored complex in alkaline medium, estimated at 650nm

Reagents
1N Folin Ciocalteau reagent, 20% Sodium carbonate, Standard Gallic Acid-100ug/ml in water

Procedure
The total phenol content was calculated using the Folin–Ciocalteu reagent assay -Aliquots of 1 mL of each extract diluted in methanol (1 mg/mL, 3 replicates per sample) had 500 µL of Folin–Ciocalteu and 6 mL of distilled water added to them. The mix was agitated for 5 min, and then 1.5 mL of Na2CO3 (20%) and 1.9 mL of distilled water were added while shaking to homogenize the dilution. The blank was prepared by substituting the same amount of diluted extract with methanol. After incubation in the dark for 2hrs, the absorbance was measured at 760nm in Spectrophotometer. All samples were analyzed in triplicates.

The results were expressed in milligrams equivalents of Gallic acid per milligram of dry weight and milligram equivalents of Quercetin per milligram of dry weight. The calibration lines were established using 0.001, 0.005, 0.01, and 0.02 mg/mL of Gallic acid and Quercetin respectively. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of extract using the formula, C= cV/M ---Where

C=total content of phenolic compounds in mg/g GAE,
c=concentration of gallic acid (mg/ml),
V=Volume of extract,
m=the weight of pure plant extract

The value was obtained by calculating the mean of three readings ± Standard deviation.

2.Estimation of DPPH
The scavenging activity of leaf extract was evaluated spectroscopically using the DPPH radical assay. The scavenging of free radicals by DPPH is defined as the percent radical scavenging activity. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. Quantitative assay was performed with stock solutions (10 mg mL^{-1}) of the plant extracts, they were prepared in methanol from which serial dilutions were carried out to obtain concentrations of 50, 100,150 and 200 mg mL^{-1}. Diluted solutions (2 mL) were added to 2 mL of a 0.004% ethanol solution of DPPH, mixed and allowed to stand for 30 min for reaction to occur. Ascorbic acid was used as positive control.

The antioxidant effect of *Diospyros ferrea* leaf using DPPH (diphenyl-1-picrylhydrazyl) using ultraviolet (UV) Spectrophotometry at 517 nm. 2 mg of leaf compound was dissolved in 100 mL of methanol to prepare a stock solution. Dissolve 9.5 g of DPPH in 25 mL of methanol to make 1mM DPPH solution. Take 1 mL of DPPH solution and mixed with 4 mL of the sample solution in methanol (comprising 5–10 µg/mL and control). All the samples were maintained in the dark for 30 minutes and absorbance was recorded at 517 nm with the help of UV Spectrophotometry. The decrease in the DPPH absorbance states an increase in the DPPH radical scavenging effect. The scavenging of free radicals by DPPH is defined as the percent radical scavenging activity.

Principle
DPPH radical is scavenged by antioxidants through the donation of a proton from the reduced DPPH. The color change from purple to yellow after reduction can be quantified by its decrease in absorbance at wavelength 517 nm.
Reagents 0.2 mM DPPH 2,2 Diphenyl-1-picryl-hydrazyl

Procedure
Take 2 mg of MLEDF dissolved in 100 mL of methanol o prepare a stock solution. Similarly, to prepare 1 mM solution of DPPH, by dissolving 9.5 g of DPPH in 25 mL of methanol. Take 1 mL of DPPH solution and mixed with 4 mL of the sample solution in methanol, comprising 5–10 µg/mL and control. All the samples were maintained in the dark for 30 minutes and absorbance was recorded at 517 nm with the help of UV spectrophotometry. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

The capability to scavenge the DPPH radical was calculated using the following equation.

DPPH radical scavenging activity (%) = Control – Sample X 100

3.Estimation of SOD:

This method utilizes the inhibition of auto-oxidation of pyrogallol by superoxide dismutase (SOD) enzyme (Sun et al 1988). The assay mixture in a 3 ml volume consisted of 100 µL each of 0.2 mM pyrogallol, 1 mM EDTA, 1 mM DTPA, and varying concentrations of standard SOD enzyme or 100 µL of serum in air equilibrated tris-HCl buffer (50 mM; pH 8.2). The reaction mixture prepared in 3 sets includes standard, test and control. Pyrogallol was added after the addition of all other reagents to start the reaction. Initial 10 s period was considered as induction period of the enzyme. After 10 s, change in absorbance at 420 nm at 10 s intervals was recorded to a period of 4 min. The average change in the absorbance per minute was calculated. One unit of enzyme SOD was defined as the amount of enzyme received to cause 50% inhibition of pyrogallol auto-oxidation, the activity of the enzyme in different standards was expressed in units/ml.

4.Estimation of Glutathion peroxidase (GSH)

This method is based on the development of a yellow color when DTNB (Ellman's Reagent) is added to Sulpha hydryl compounds due to redox reaction between GSH and DTNB. The color which develops is fairly stable for about 10 min, and the reaction is little affected by variation in temperature. The reaction is read at 412 nm. GSH in red cells is relatively stable and venous blood samples anti coagulated with ACD maintain GSH levels up to 3 weeks at 4°C GSH is slowly oxidized in solution, so only fresh lysates should be used for the assay.

Whole blood (200 µl) is mixed thoroughly with 1.8 ml of distilled water and 3 ml of precipitate allows standing for 5 min and filtered. Two test tubes were taken ,one is test and other is blank. In test marked test tube, 2 ml of clear filtrate added from the above mixture to 8 ml of disodium phosphate buffer and 1 ml of DTNB reagent added to it and mixed well. The color developed rapidly, stable for 10 min. A reagent blank was made using 2 ml of distilled water, 8 ml of phosphate buffer, and 1 ml of DTNB reagent. Readings were taken at 412 nm in the Spectrophotometer.

5.Estimation of Catalase

The method is based on the fact that dichromate in acetic acid gets reduces to chromic acetate when heated in the presence of H_2O_2 with the formation of per chromic acid which is an unstable compound. The chromic acetate thus produced is measured by Colorimetry at 570 nm. The Catalase (CAT) preparation allows splitting of H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate or acetic acid mixture, and the remaining H_2O_2 is determined by measuring chromic acid with Colorimetry after heating.

Three sets of tubes were arranged and labeled as blank, test (0 s) and test (60 s), and proper reagent additions were made. The tubes were boiled for 10 min, cooled to room temperature and readings were taken at 570 nm. Different concentrations of H_2O_2 ranging from 10 to 160 µmoles were taken in tubes and preceded. The activity of CAT was expressed as units/ml of the serum sample. One unit of CAT activity represents the amount of enzyme that destroys 1 µmole H_2O_2/min.

Statistical analysis

All values are expressed as Mean ± S.E.M. The data was analyzed for ANOVA and post hoc Dennett's t- test. The results were considered statistically significant when $p<0.05$. The statistical analysis was carried out using Graph pad in stat 3.0 software and antioxidant values are expressed ± SD

RESULTS & DISCUSSION

High spectrum of GC-MS using database pattern of *Diospyros ferrea* (willd) Bakh, methanolic leaf extract under mobile phase through carrier gas showed beta-Sitosterol, Lupeol, Betulin, alpha-Amyrin, β-amyrin, Isodiospyrin, Quercetin, Ursolic & Betulinic acids, Kaempferol, Phytol, Friedelan-3-one, Squalene, Citronellol, Pregnenolene, Olen, Thunbergol and tochopherol which are grouped under phenolics, hydrocarbons, terpenes of potent compounds.

Solvent -1 Hexane	Solvent-2 Ethyl acetate	Solvent-3 Methanol
7 Compounds	15 Compounds	17 Compounds
Phenol 2,4 bis(1,1-dimethyl-alkylated phenolic group	Phytol-diterpene-Fragrance alcohol	Phenol
All peaks are siloxone - hydrocarbons	Squalene-Hydrocarbons-Triterpene	Galactitol-sugar alcohol
	Di-alpha-Tocopherol-E	Squalene-Hydrocarbons Triterpene
	Alpha-Amyrin-Triterpene	alpha-Amyrin-Triterpene
	Friedelan-3-1,Triterpene	All peaks are siloxone - hydrocarbons

Table1: Type of solvent extract GC-MS D.ferrea leaf extract

RT	Area%	Norm%	MW	NAME	Chemical formula
17.216	1.900%	6.34	280.0	17-Octadecynoic acid	C18H32O2
			254.0	E-9-Methyl-8-tridecen-2-ol,acetate	C16H30O2
			168.0	7-Oxybicyclo[4.1.0]heptane,1-methyl-4-(2-methyloxiranyl)-	C10H16O2
17.996	1.116	3.73	238.0	1-(7-Hydroxy-1,6,6-trimethyl-10-oxatriclo[5,2,1,0(2,4)]dec-9-yl)ethanone	C14H22O3
			222.0	3-Buten-2-one,4-)2,5,5-trimethyl-3,8-dioxatricyclo[5,1,0,0,(2,4)]oct-4-yl-	C13H18O3
			268.0	7-Methyl-Z-tetradecen-1-ol acetate	C17H32O2
18.063	1.067	3.56	224.0	2-Pentanone,4-(1,3,3-trimethyl-7-oxabicyclo[4,1,0]hept-2-yl)-	C14H24O2
			444.0	9-Octadecenoic acid, (2-phynyl-1,3-dioxolan-4-yl) methyl ester, trans-	C28H44O4
			266.0	5a-methoxy-9a-3,4,5a,6,7,8,9a,10-octahydropyrano[4,3-b]chromene-1-9-dione	C14H18O5
18.310	2.356	7.87	496.0	9,12,15-Octadecatrienoic acid,2[(trimethylsilyl)oxy]1-oxymethyl ester-	C27H52O4Si2
				2-(2-(2-Butoxyethoxy)ethoxy)ethyl3-methylbutanoate	C15H30O5
				2-(2-Butoxyethoxy)ethoxy)ethyl3-methylbutanoate	C13H26O4

RT	Area%	Norm%	MW	Name	Chemical formula
19.190	29.940	100.0	182.0	Galactitol	C6H14O6
			182.0	Sorbitol	C6H14O6
			210.0	d-Glycero-l-gluco-heptose	C7H14O7
19.417	20.007	66.82	266.0	1-Nonadecene	C19H38
				9-Eicosene	C20H40
				1-Docosene	C22H44O2
20.170	2.265	7.56	364.0	Eicosane,2-cyclohexyl	C26H52
			238.0	Undecene,2- cyclohexyl	C17H34
			350.0	2-cyclohexylnonadecane	C25H50
20.530	3.366	11.24	296.0	Phytol	C20H40O
			296.0	Isophytol	C20H40O
			296,0	Phytol	C20H40O
21.204	0.841	2.81	256.0	Eicosyl acetate	C22H44O2
26.072	1.090	3.64	222.0	Fernesol isomers	C15H26O
28.112	3.122	10.43	472.0	Alpha Tocopheryl acetate	C31H52O3
29.099	1.390		396.0	Stigmastan -6,22-dien,3,5-dodihydro-	C29H46
29.579	1.712		316.0	Pregnenolone	C21H32O2
29.966	2.671		410.0	Olen-12-en	C30H50O
30.406	1.287		468.0	Urs-12-en-24-oige acid,3-oxomethyl ester	C31H48O3
31.553	4.381		290.0	Thunbergol	C20H34O
31.840	0.817		428.0	D:A Fridooleanan-28-1,3-oxo	C30H52O

Table 2: GC-MS screening of methanolic leaf extract of D. ferrea

Concentration of MLE of *D.ferrea*	% of Phenol	Total phenolic content in Methanol (mg GAE/g dry extract)
100 mg	25.6 GAEs/g	37.54 ± 2.30
200	45.86	42.86 ± 1.75
300	72.8	55.03 ± 0.42
400	88.96 GAEs/g	66.14± 0.22

Table 3: Total Phenolic content of Leaf extract of *D. ferrea*

The antioxidant activity of each extract at a particular concentration was expressed as the number of micrograms equivalent of ascorbic acid that was calculated by keeping absorbance values of test extracts in the linear regression equation of calibration curve of antioxidant activity of ascorbic acid at known concentrations and vit E at 20 µg/ml, 40 µg/ml, 80 µg/ml, and 160 µg/ml respectively.

MLEDF µg/ml Scavenging ability and Absorbance concentration	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml
µg/ml-	18.206 ± 0.25	37.1802 ± 0.56	54.2039 ± 1.0	89.3405 ± 1.67
Absorbance	0.103 ± 0.06	0.225 ± 0.04	0.49 ± 0.48	0.942 ± 0.03

Values are represented as mean ± standard error of triplicate experiment

Table 4: DPPH radical scavenging ability of phenol in the leaf extract of D. ferrea

DPPH results were expressed at different concentrations ie., 50,100,150 µg/ml 60±0.17, 64±0.14, 68±0.33 µg/ml in DPPH antioxidant assays, has been found best method to evaluate phenolic compounds and Tocopherol. Hydrogen donation can reduce the 2,2-diphenyl-1-picrylhydrazyl and DPPH radical is converted to the corresponding hydrazine and the color of the solution is changed from violet to yellow indicates the scavenging behavior of the leaf sample understood

Concentration mg mL^{-1}	Inhibition ±SEM(%) MLE *D. ferrea*	Inhibition ±SEM(%) Tocopherol
50	60±0.17	30.60±0.11
100	64±0.14	43.90±0.88
150	68±0.33	54.41±1.12

Table:5. DPPH Radical scavenging activity of leaf extract *D. ferrea*

Test	Absorbance at 517nm	Percent of Antioxidant
Control	-	0.682nm
Sample	40.1220	0.554 nm

% of antioxidant activity = [(A control – A sample) ÷ Ac] × 100
[(0.682 − 0.554) ÷ 0.682] × 100 40.1220

Results were showed significant increase in the catalase and glutathione levels of the leaf extract in treated group compared to untreated group of in vivo antioxidant potentials. Significantly increased antioxidant enzymes levels in MLEDF noticed which is a potential source of antioxidant property of the leaf material. Further studies are required to explore the therapeutic identification and use.

Group	Superoxide dismutase SOD (IU/L)	Lipid peroxidase (IU/L)	Glutathione GSH(IU/L)	Catalase (IU/L)
Normal control –I	190.5±1.118	95.83 ± 1.537	107 ± 1.528	18.5 ± 0.428
Diabetic control-II STZ 60mg/kg	131.83±0. 945+++	170.166 ± 2.822 ***	76 ± 1.291 +++	8.5 ± 0.428 +++
Standard drug- III Glibenclamide 10 mg/kg	187.01±1. 065***	100.66 ± 0.714 ***	102.83 ± 1.078 ***	17.33 ± 0.333***
Treatment lower dose MLE of *D.ferrea*- 200mg/kg bw- IV	153.33±1. 498***	132.66 ± 1.145 ***	89.83 ± 1.302 ***	11.83 ± 0.307***
Treatment lower dose MLE of *D.ferrea* 400mg/kg bw- V	180.33±1. 282***	100.66 ± 1.145 ***	94.5 ± 0.670 ***	14.83 ± 0.307 ***

All values were expressed as Mean ± SEM. n =6

Table 7: Effect of Methanol leaf extract of D. ferrea on antioxidant enzymes

Animals treated with 200 and 400 mg/kg doses of D.ferrea leaf extract manifested glutathione levels 89.83 ± 1.302, 94.5 ± 0.670 respectively. All the results indicate that leaf extract significantly protected the rats from H_2O_2-induced oxidative stress as its antioxidant effect is dose-related.

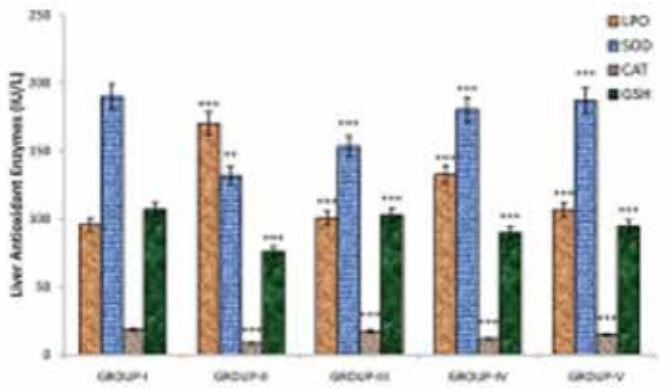

Fig 2 : Effect of MEDFL on Liver Antioxidant Enzymes

The DPPH radical scavenging assay proves that methanol extract has shown the potential effect on inhibiting the free radicals in dose dependent manner. Hydroxyl free radicals are generated by H2O2 in tissues which are highly reactive metal ions can attack DNA that causes lipid peroxidation, tissue damage, protein denaturation and glutathione depletion easily. So, Reactive oxygen species includes singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl and non-oxygen radicals are released.

Table 7 showed a significant decrease in the levels of SOD was observed in the diabetic control group, when compared to the normal group I. The group-III receiving standard drug had significant increase in the SOD levels, when compared to the diabetic control group II. The groups-IV and V treated with MLEDF 200 and 400 mg/Kg also exhibited a significant increase in the SOD levels, when compared to the diabetic control group II.

A significant decrease in GSH levels was observed in the diabetic control group, when compared to the normal group I. The group-III receiving standard drug had significant increase in the pancreatic GSH levels, when compared to the diabetic control group II. The groups-IV and V treated with MLEDF 200 and 400 mg/Kg also exhibited a significant increase in the pancreatic GSH levels, when compared to the diabetic control group II.

A significant decrease in the levels of Catalase was observed in the diabetic control group, when compared to the normal group I. The group-III receiving standard drug had significant increase in the catalase levels, when compared to the diabetic control group II. The groups-IV and V treated with MLEDF 200 and 400 mg/Kg also exhibited a significant increase in the catalase levels, when compared to the diabetic control group II. Glutathione, a dimeric tripeptide with a thiol group reduced to monomeric form, and it works as powerful endogenous antioxidant, protecting biological systems from degenerative damages. Glutathione participates in leukotriene synthesis and acts as a co-factor for the enzyme glutathione peroxidase synthesis, that scavenges H2O2 and other peroxides from the body.

A significant increase in the levels of LPO was observed in the diabetic control group, when compared to the normal group I. The group-III receiving standard drug had significant decrease in the LPO levels, when compared to the diabetic control group II. The groups-IV and V treated with MLEDF 200 and 400 mg/Kg also exhibited a significant decrease in the LPO levels, when compared to the diabetic control group II.

Excessive lipid peroxidation among Group I and II animals, confirmed the ability of H2O2, at the dose used, to induce oxidative stress in animals. DF leaf extract significantly inhibited lipid peroxidation and glutathione depletion arising from H2O2-induced oxidative stress. Its antioxidant activity is probably mediated through chain breaking which leads to reduction of free radicals. Vitamin E –Tocopherol shares a similar mechanism known as carotenoids (β -carotene) are usually components of lipids (Harborne et al., 1998).

CONCLUSION

The current study of *D.ferrea* leaf is a good therapeutic source of antioxidants which reveals the presence of potent antioxidant phytochemicals significantly reduced oxygen stress and free radicals and enhance defense against damage.

1. Methanolic leaf extract of *D.ferrea* of polyphenolic compounds and Betulin showed an excellent antioxidant activity and highly beneficial to prevent free radicals and inflammation in the body.The presence of hydroxyl groups of polyphenolic compounds would be expected to enhance solubility of organic compounds. Methanolic extract of *D.ferrea* exhibit anti-oxidative potential may be due to chelating property of phenols.
2. DPPH is a rapid established quantitative antioxidant assay to evaluate the scavenging activity accurately. The activity of SOD, CAT and GSH enzymes in 200mg/kg and 400mg/kg treated Wistar rats with *D.ferrea* leaf extract was significantly neutralize the free radicals by inhibiting through phagocytosis and preventing the formation of OH and LPO. So, *D.ferrea* leaf extract showed best antioxidant property. The purpose of phenolic compounds of plants is total protection against UV radiation stress.

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